Comparison of the binding affinities of acyl-CoA-binding protein and fatty-acid-binding protein for long-chain acyl-CoA esters

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Bovine and rat liver acyl-CoA-binding proteins (ACBP) were found to exhibit a much higher affinity for long-chain acyl-CoA esters than both bovine hepatic and cardiac fatty-acid-binding proteins (hFABP and cFABP respectively). In the Lipidex 1000- as well as the liposome-binding assay, bovine and rat hepatic ACBP effectively bound long-chain acyl-CoA ester. h- and c-FABP were, under identical conditions, unable to bind significant amounts of long-chain acyl-CoA esters. When FABP, ACBP and [1-¹⁴C]hexadecanoyl-CoA were mixed, hexadecanoyl-CoA could be shown to be bound to ACBP only. The experimental results give strong evidence that ACBP, and not FABP, is the predominant carrier of acyl-CoA in liver.

INTRODUCTION

Fatty-acid-binding proteins (FABP) form a family of low- M_r [(14–15) × 10³] proteins which bind fatty acids and are believed to be involved in their intracellular transport and metabolism (Bass, 1985; Glatz & Veerkamp, 1985; Glatz *et al.*, 1987; Spener *et al.*, 1989). The most studied FABP is the liver type (hFABP) (Bass, 1985). Cardiac FABP (cFABP) differs clearly from hFABP immunologically, and these two proteins also differ from the intestinal type (iFABP) (Bass, 1985). A number of studies have shown that hFABP also binds long-chain acyl-CoA esters (Bass, 1985; Glatz & Veerkamp, 1985; Burrier *et al.*, 1987). The formation of acyl-CoA is the initial step in fatty acid utilization in the cell. Acyl-CoA partitions readily into membranes (Lichtenstein *et al.*, 1982) and is synthesized by enzymes localized in mitochondrial and microsomal fractions (De Jong & Hülsmann, 1970). Acyl-CoA also has detergent properties and inhibits membrane-associated enzymes, including methylsterol oxidase, hydroxymethylglutaryl-CoA reductase and acyl-CoA cholesterol acyltransferase (Grinstead *et al.*, 1983), as well as acetyl-CoA carboxylase (Lunzer *et al.*, 1977) and mitochondrial adenine nucleotide translocase (Babour & Chan, 1979). The inhibition of all these enzymes by acyl-CoA could be relieved by addition of hFABP, presumably due to acyl-CoA binding by hFABP.

Acyl-CoA is believed to have general damaging effects in membranes (Brecher, 1983). It has been suggested that hFABP plays an important role in protecting membranes from these damaging effects of acyl-CoA esters (Burrier *et al.*, 1987). The same authors reported that rat hFABP had two independent binding sites, one for fatty acid and one for acyl-CoA esters. Furthermore the authors suggested that hFABP could influence both synthesis and degradation of acyl-CoA. In contrast with rat hFABP, they found that rat cFABP was unable to bind acyl-CoA esters. This questions the general role of FABP in acyl-CoA metabolism and membrane protection.

Recently a new lipid-carrier protein able to bind acyl-

CoA esters, but not fatty acids, has been discovered. This protein, named 'acyl-CoA-binding protein' (ACBP) has been isolated and characterized from bovine and rat liver. The M_r values are 9955 and 9932 respectively, and the proteins show no sequence similarity to any known FABPs (Mikkelsen & Knudsen, 1987; Mikkelsen *et al.*, 1987; Knudsen *et al.*, 1989). The protein is mainly cytosolic, and the highest concentration is found in liver. It has been shown to bind saturated acyl-CoA esters with chain lengths from 8 to 18 carbon atoms with high affinity, but is unable to bind free fatty acids (Mikkelsen & Knudsen, 1987; Knudsen *et al.*, 1989). The physiological role of ACBP is unknown and, in addition to its potential role in acyl-CoA metabolism, the protein may have neurotransmitter activity, as the sequence of bovine and rat brain diazepam-binding inhibitor is identical with the sequence of bovine and rat liver ACBP respectively (Knudsen *et al.*, 1989).

The finding of a new acyl-CoA-binding protein raises the question of which of the two proteins, hFABP or ACBP, is the carrier protein for acyl-CoA esters. An important factor in this connection is the relative binding affinity of the two proteins for acyl-CoA esters. In the present paper, therefore, we compare the relative binding affinity for acyl-CoA esters of bovine hFABP and cFABP with that of bovine and rat ACBP. The result shows that ACBP has a much higher affinity for acyl-CoA esters than both the FABP types tested.

MATERIALS AND METHODS

Enzymes and special reagents

Sprague–Dawley rats (200 g) were used, and cow livers were obtained from the local slaughterhouse. Sephadex G-50, Sephadex G-100 material and Q-Sepharose-HP and S-Sepharose-HP columns (XK16) were from Pharmacia, Uppsala, Sweden. Lipidex 1000 was obtained from Packard Instrument Co., Downers Grove, IL, U.S.A. and *cis*-9-[1-¹⁴C]octadecenoic acid, [1-¹⁴C]hexa-

Abbreviations used: ACBP, acyl-CoA-binding protein; cFABP, hFABP and iFABP, cardiac, hepatic and intestinal fatty-acid-binding protein respectively.

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decanoic acid and 1,2-dipalmitoyl L-3-phosphatidyl[*N*-methyl-³H]choline, were from Amersham International p.l.c. All other chemicals used were of analytical grade.

Purification of ACBP

ACBP was purified from bovine and rat liver as described by Mikkelsen *et al.* (1987) for the purification of bovine liver ACBP.

Purification of hFABP

FABP from bovine liver was purified as described by Schulenberg-Schell *et al.* (1988) or by the method described above for ACBP, with the following modifications: the Sephadex G-50 column was run in 30 mM-Tris/HCl, pH 9.0. The fractions containing FABP were pooled and incubated with 1 mg of *cis*-9-octadecenoic acid. The pooled FABP-fatty acid complex was loaded on to a Q-Sepharose-HP column (10 cm × 1.6 cm) and the proteins were eluted with a linear gradient of NaCl (0–100 mM for 120 min; flow rate 4 ml/min). The FABP-containing fractions were further purified by gel-filtration chromatography on a Sephadex G-50 column (80 cm × 1.5 cm) equilibrated and eluted with 20 mM-potassium phosphate (pH 7.0)/140 mM-NaCl.

Purification of bovine cFABP

cFABP was purified as described by Jagschies *et al.* (1985).

Electrophoresis

All ACBP and FABP preparations used were analysed on a Pharmacia Phast System with 20%-(w/v)-acrylamide gels as recommended by the manufacturer. In this system FABP and ACBP could be clearly separated.

Synthesis of long-chain acyl-CoA

cis-9-[1-¹⁴C]Octadecenoic or [1-¹⁴C]hexadecanoic acid (250 µCi, ~ 5 µmol) in toluene was evaporated to dryness with a gentle stream of N₂ in the vial in which they were supplied from Amersham. Methylene chloride (1.0 ml) and equimolar amounts of triethylamine were added and the sample was incubated for 30 min at room temperature. After cooling to 0 °C, an equimolar amount of ethyl chloroformate were added and the incubation was continued for 2 h. The methylene chloride was evaporated with N₂, and the dry residue was dissolved in 1.5 ml of *t*-butanol. The mixed anhydride in *t*-butanol was mixed with 10 µmol of CoASH dissolved in 1.5 ml of 0.4 M-KHCO₃, pH 8.3, and incubated for 10 min at room temperature. The pH was decreased to 1.0 with 2 M-HCl and evaporated on a rotary evaporator. The dry residue was extracted with 3 × 1 ml of diethyl ether, 3 × 1.0 ml of acetone and 3 × 1.0 ml of 0.1 M-HCl. The precipitate was resuspended in 2.0 ml of water and dissolved by adjusting the pH to 3.5 with drops of 0.5 M-NaHCO₃. Acyl-CoA was again precipitated by decreasing the pH to 1.0 with HCl. The suspension was extracted with 2 ml of diethyl ether four times in order to extract the last traces of free acid. The pH was again adjusted to 3.5, and the concentration of acyl-CoA was calculated from u.v. absorption at 260 nm by using a molar absorption coefficient (ϵ_{260}) of 15.4 litre · mmol⁻¹ · cm⁻¹. The acyl-CoA was analysed by t.l.c. on cellulose plates as described by Pullman (1973). The developed plates were scanned on a Berthold Automatic t.l.c. linear analyser and were shown

not to contain non-esterified fatty acids. The yield in the synthesis was about 40 % (calculated from the amount of radiolabelled fatty acid used). The acyl-CoA esters were stored at pH 3.5 at -20 °C.

Preparation of liposomes

The designated amount of lipid for the liposomes was dissolved in chloroform/methanol (2:1, v/v). The solvent was evaporated with N₂, and the lipid was further dried in a vacuum centrifuge for 60 min at room temperature. The lipid mixture was resuspended in 10 mM-Tris/HCl (pH 7.4)/0.1 M-NaCl (buffer A) by gentle agitation for about 3 h at ambient temperature. Stock solutions of liposomes with radiolabelled hexadecanoic acid (sp. radioactivity 1.0 Ci/mol) contained 40 mM-phosphatidylcholine, 13 mM-cholesterol and 2 mM-hexadecanoic acid. Stock solution of liposomes containing [1-¹⁴]hexadecanoyl-CoA (sp. radioactivity 55 Ci/mol) and *cis*-9-[1-¹⁴C]octadecenoyl-CoA (sp. radioactivity 42.6 Ci/mol) contained 36 mM-PtdCho, 12 mM-cholesterol and 18 µM CoA ester. Known amounts of 1,2-dipalmitoyl L-3-phosphatidyl[*N*-methyl-³H]choline were included in order to monitor the content of phospholipid in stock solutions and to be able to correct for radiolabelled acyl-CoA in unsedimented liposomes during the assay. Before use, an aliquot of the stock liposome solution was diluted 1:4 (v/v) with buffer A and centrifuged at 18000 *g* for 2 min. The supernatant containing the smallest multilamellar liposomes and vesicles was discarded and the pellet was gently resuspended and used in the binding assay.

Liposome binding assay

The incubation (total 800 µl) was carried out in 1.5 ml Eppendorf tubes. Acyl-CoA-containing liposomes were diluted to the appropriate concentration in 600 µl of buffer A, and the indicated amount of binding protein was added in 200 µl of buffer. A. When fatty acid-containing liposomes were used, the assay was scaled down to a final total volume of 400 µl. After addition of the binding protein and gentle mixing, the samples were incubated for 60 min at room temperature and centrifuged at 12000 *g* for 10 min in a Sigma 2MK centrifuge; 75% of the supernatant was transferred to 4.0 ml of scintillation liquid for determination of radioactivity.

The amount of ligand present in the supernatant was corrected for ¹⁴C-labelled ligand in unsedimented liposomes. This value was calculated from the amount of 1,2-dipalmitoyl L-3-phosphatidyl[*N*-methyl-³H]choline remaining in the supernatant, assuming that the ³H/¹⁴C ratio was unchanged. It was found to be ≤ 3 % of the radioactive ligand added.

Lipidex 1000 binding assay

The binding assay with [1-¹⁴C]hexadecanoyl-CoA (sp. radioactivity 10 Ci/mol) and *cis*-9-[1-¹⁴C]octadecenoyl-CoA (sp. radioactivity 10 Ci/mol) was performed in 1.5 ml Eppendorf tubes in a final vol. of 200 µl. The indicated amount of acyl-CoA was diluted in 150 µl of 10 mM-potassium phosphate buffer, pH 7.4 (binding buffer). Binding protein to give a final concentration of 0.2 µM was added in 50 µl of the above buffer. The samples were mixed and incubated at 37 °C for 30 min, chilled on ice for 10 min and mixed with 400 µl of an ice-cold 50 % slurry of Lipidex 1000 in binding buffer. After 10 min incubation on ice, the samples were centrifuged at

12000 *g* for 5 min at 0 °C, and 200 μ l of the resulting supernatant was transferred to 4 ml of scintillation solution for determination of radioactivity. The assay was carried out in triplicate, and blanks were run for each concentration to ensure that all unbound ligand could be completely removed from the incubation medium by the Lipidex at all ligand concentrations used.

Determination of the relative binding affinity of acyl-CoA to ACBP and hFABP by Sephadex G-50 gel-filtration chromatography

FABP and ACBP were incubated with [1^{14} C]hexadecanoyl-CoA or *cis*-9-[1^{14} C]octadecenoic acid in 1.0 ml of 10 mM-potassium phosphate (pH 7.4/140 mM-NaCl) and loaded on to a column (1.25 cm $\times$ 285 cm) of Sephadex G-50 (Superfine grade) equilibrated with the same buffer. The flow rate was 6.5 ml/h, and the eluate was monitored at 227 nm. Fractions (1.0 ml each) were collected for the determination of radioactivity.

Protein determination

The concentration of ACBP was calculated by amino acid analysis (Knudsen *et al.*, 1989) or by uv-absorption at 280 nm. The $A_{280}^{1\%}$ for bovine ACBP was determined to be 15.5 from amino acid analysis. The concentration of bovine hFABP was determined by u.v. absorption at 280 nm [using 3.0 as the $A_{280}^{1\%}$ (Schulenberg-Schell *et al.*, 1988)] or by weighing of freeze-dried material. The $A_{280}^{1\%}$ used for measuring cFABP was 8.1 (Jagschies *et al.*, 1985). Protein in microsomal membranes was determined by the method described by Schachterle & Pollack (1973).

Preparation of rat liver microsomal membranes

Rat liver was homogenized in 4 vol. of 0.25 M-potassium phosphate buffer (pH 7.4)/1 mM-EDTA with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 14000 *g* for 15 min at 4 °C, the precipitate was discarded and the supernatant further centrifuged at 100000 *g* for 60 min. The precipitate was resuspended in 10 mM-Tris/HCl (pH 7.4)/100 mM-NaCl and stored at -80 °C.

Binding of acyl-CoA to microsomal membranes

Microsomal membranes were heated at 85 °C for 15 min to inhibit hydrolysis. The heated membranes were incubated for 10 min at room temperature with 5 μ M-*cis*-9-[1^{14} C]octadecenoyl-CoA $\pm$ 5 mM bovine hFABP or ACBP in a total vol. of 85 μ l of 10 mM-Tris/HCl (pH 7.4)/100 mM-NaCl and then centrifuged at 130000 *g* for 90 min at 4 °C in a Beckman Airfuge. A 50 μ l portion of the resulting supernatant was transferred to a 4 ml liquid-scintillation cocktail for determination of radioactivity. Hydrolysis of *cis*-9-[1^{14} C]octadecenoyl-CoA was measured as described by Burrier *et al.* (1987) and was found to be less than 10% in the incubation containing microsomal membrane alone and lower when FABP or ACBP was present.

RESULTS AND DISCUSSION

FABP from bovine heart and liver exhibited much lower affinity for both [1^{14} C]hexadecanoyl-CoA and *cis*-9-[1^{14} C]octadecenoyl-CoA than bovine and rat liver ACBP, when binding was compared under identical conditions in the Lipidex 1000 binding assay (Figs. 1a and 1b). The K_d values calculated from the Scatchard

Fig. 1. Binding of *cis*-9-octadecenoyl-CoA and hexadecanoyl-CoA to bovine and rat ACBP, bovine hFABP, cFABP and serum albumin

A binding assay employing Lipidex 1000 was performed by using 0.2 μ M-ACBP, -FABP and -serum albumin respectively. (a) Hexadecanoyl-CoA; (b) *cis*-9-[1^{14} C]octadecenoyl-CoA. Results are means of triplicates; the s.d. values are shown by bars. The assay was carried out as described in the Materials and methods section. ■, Bovine hFABP, □, bovine cFABP; ●, bovine ACBP, ○, rat ACBP; and △, bovine serum albumin.

plots for [1^{14} C]hexadecanoyl-CoA binding were 0.22 μ M and 0.27 μ M for bovine and rat ACBP respectively (Figs. 2a and 2b). The K_d values for *cis*-9-[1^{14} C]octadecenoyl-CoA binding were 0.14 μ M and 0.13 μ M respectively (Figs. 2a and 2b). The binding stoichiometry calculated from Scatchard plots for hexadecanoyl-CoA binding is 0.95 mol/mol and 1.02 mol/mol for bovine and rat ACBP respectively, the values for *cis*-9-octadecenoyl-CoA binding were 0.95 mol/mol and 0.87 mol/mol respectively. These values are in agreement with previously estimated values from binding studies based on fluorescence measurements (Mikkelsen & Knudsen, 1987; Knudsen *et al.*, 1989). Bovine hFABP and cFABP exhibited much lower binding than ACBP. Furthermore, hFABP bound acyl-CoA esters only slightly better than cFABP. The binding of acyl-CoA esters by both FABP species was so low that no meaningful K_d could be calculated.

To exclude the possibility that the lack of binding of acyl-CoA by the bovine cFABP and hFABP used in the above experiment was due to the use of inactive FABP preparations, we tested the ability of these two proteins to bind fatty acids.

Fig. 2. Scatchard plots of the binding data for hexadecanoyl-CoA and *cis*-9-octadecenyl-CoA binding in Fig. 1

(a) Bovine ACBP, (b) rat ACBP. ●, *cis*-9-Octadecenyl-CoA; ○, hexadecanoyl-CoA.

Fig. 3. Binding of hexadecanoic acid to bovine hFABP and cFABP

Binding assays employing [1-¹⁴C]hexadecanoic acid-loaded liposomes were performed using 1.25 μ M-FABP. Results are means of duplicates ($\pm$ half the difference between duplicates is shown by the bars). The assays were carried out as described in the Materials and methods section. ■, Bovine hFABP; □, bovine cFABP.

Both proteins bound fatty acid when tested in the liposome assay (Fig. 3). The ability of the FABP preparations used to bind fatty acids was further confirmed by using Sephadex G-50 gel-filtration chromatography and Lipidex 1000 assay (results not shown). The above observed low binding of acyl-CoA esters by FABP compared with acyl-CoA binding by ACBP is therefore not likely to be due to the use of inactive FABP preparations.

The above results strongly indicate that acyl-CoA will be preferentially bound to ACBP and not to FABP in solutions with both proteins present. That this happens is also shown in Fig. 4, using Sephadex G-50 gel-filtration chromatography. Bovine ACBP and hFABP in equimolar amounts were mixed with subsaturating concentrations of either [1-¹⁴C]hexadecanoyl-CoA (Fig. 4b) or *cis*-9-[1-¹⁴C]octadecenoic acid (Fig. 4a). The concentration of ligand used was half the concentration of the

Fig. 4. Separation of bovine hFABP and ACBP previously incubated with *cis*-9-[1-¹⁴C]octadecenoic acid and [1-¹⁴C]hexadecanoyl-CoA

(a) A 10 nmol portion each of hFABP and ACBP were incubated with 5 nmol of *cis*-9-[1-¹⁴C]octadecenoic acid (sp. radioactivity 5 Ci/mol). (b) A 10 nmol portion each of hFABP and ACBP were incubated with 5 nmol of [1-¹⁴C]hexadecanoyl-CoA (sp. radioactivity 5.9 Ci/mol). The proteins were incubated with the ligands in 10 mM-potassium phosphate (pH 7.4)/140 mM-NaCl for 10 min at 37 °C and loaded on to the Sephadex G-50 column. For further details, see the Materials and methods section.

individual proteins. The two proteins were separated on a 285 cm $\times$ 1.25 cm Sephadex G-50 column. The elution position of FABP and ACBP were determined by running the proteins individually (V_e for FABP 224 ml; V_e for ACBP 232 ml). The results in Fig. 4(b) clearly show that ACBP effectively competes with FABP for acyl-CoA. Essentially all the recovered [1-¹⁴C]hexadecanoyl-CoA was located in the ACBP peak. In the *cis*-9-octadecenoic acid experiment the opposite situation occurred. The added *cis*-9-[1-¹⁴C]octadecenoic acid was found exclusively in the FABP peak (Fig. 4a).

The recovery of [1-¹⁴C]hexadecanoyl-CoA after the Sephadex G-50 column was 36%, showing that the ACBP effectively competes with binding sites on Sephadex G-50 for hexadecanoyl-CoA. The recovery of *cis*-9-[1-¹⁴C]octadecenoic acid in the bovine hFABP peak was only 1.2%. This shows that Sephadex G-50 has binding sites with similar affinity for *cis*-9-octadecenoic acid as FABP.

In rat liver the concentration of long-chain acyl-CoA has been reported to be 108–248 nmol/g of liver protein

(Woldegiorgis *et al.*, 1985). The ACBP concentration has been found to be 408 nmol/g of liver protein (Knudsen *et al.*, 1989) or 2–4-fold the long-chain acyl-CoA concentration.

The above-reported binding studies therefore strongly suggest that acyl-CoA, under conditions *in vivo* in the liver, most likely is bound to, and transported by, ACBP and not FABP. The above results also confirm that ACBP does not bind non-esterified fatty acids, as previously reported by Mikkelsen & Knudsen (1987) and Knudsen *et al.* (1989).

It is known that long-chain acyl-CoA species readily partition into membranes (Lichtenstein *et al.*, 1982). In order to function as an acyl-CoA transporter in the cell *in vivo*, ACBP has to be able to compete with membranes for long-chain acyl-CoA esters. We therefore tested the ability of ACBP to extract [14 C]hexadecanoyl-CoA and *cis*-9-[14 C]octadecenoyl-CoA from multilamellar liposomes. For comparison we also included bovine

hFABP and cFABP and bovine serum albumin in these assays (Fig. 5).

Both rat ACBP and bovine ACBP were able to extract [14 C]hexadecanoyl-CoA (Fig. 5a) and *cis*-9-[14 C]octadecenoyl-CoA (Fig. 5b) from multilamellar liposomes. The binding increased to 0.2–0.4 mol of acyl-CoA/mol of ACBP when the liposome concentration was increased to about 0.4 mM-phosphatidylcholine. Higher concentrations of liposomes increased binding only slightly. Bangham *et al.* (1967) estimated that only about 10% of phospholipid and therefore possibly also acyl-CoA is located in the outer layer of multilamellar liposomes and available for binding proteins. Assuming that this value is correct, ACBP effectively removes most of the available hexadecanoyl-CoA from the liposomes at low liposome concentration.

Neither the two FABPs nor bovine serum albumin could extract significant amounts of acyl-CoA from the membrane. This indicates that only ACBP and not FABP effectively protects membranes against damaging effects of long-chain acyl-CoA esters. The above binding studies are representative and have been repeated with a number of ACBP and FABP preparations.

Burrier *et al.* (1987) found a similar low extraction of *cis*-9-octadecenoyl-CoA by rat cFABP from multilamellar liposomes with the same acyl-CoA loading and composition as used in the present experiment. However, those authors found that rat hFABP was able to extract acyl-CoA from multilamellar liposomes. In the present experiment we were unable to detect any significant difference in the ability of bovine cFABP and hFABP to extract acyl-CoA esters from liposomes. The explanation for the discrepancy between the present results and the results of Burrier *et al.* (1987) could be that bovine hFABP and rat hFABP are different or that the preparations of rat hFABP used by Burrier *et al.* (1987) were contaminated with ACBP. In our experience it is difficult to completely separate the two proteins by the methods used by Burrier *et al.* (1987); furthermore, those authors did not document the purity of the FABP preparations used. They also compared rat cFABP and hFABP in the Lipidex assay, and here again they found the same difference between hFABP and cFABP as observed in the liposome assay. A serious complication in comparing the data obtained with the Lipidex assay by Burrier *et al.* (1987) and the data obtained with the same assay in the present experiment is a significant difference in the assay conditions used. We worked in the concentration range 0–1.0 μ M-acyl-CoA and a minimum Lipidex/acyl-CoA ratio of 1000 μ l/nmol. Burrier *et al.* (1987) used 0–20 μ M-acyl-CoA and a minimum Lipidex/acyl-CoA ratio of 10 μ l/nmol at the highest acyl-CoA concentration employed. If we used a lower Lipidex/acyl-CoA ratio than 1000 μ l/nmol the Lipidex could not remove all the acyl-CoA from incubations without proteins added. We have therefore studied high-affinity binding only, whereas Burrier *et al.* (1987) have studied high- and low-affinity binding, owing to the presence of free acyl-CoA in the incubation mixture after removal of Lipidex by centrifugation. The difference between the binding ability of rat cFABP and hFABP in experiments reported by Burrier *et al.* (1987) could be that hFABP is different from cFABP with regard to low-affinity sites or that hFABP is contaminated with ACBP.

In the experiments reported by Burrier *et al.* (1987) the authors also claimed that rat hFABP contained two

Fig. 5. Binding of *cis*-9-octadecenoyl-CoA and hexadecanoyl-CoA to bovine and rat ACBP, bovine hFABP, cFABP and serum albumin

Binding assays employing radiolabelled acyl-CoA-loaded liposomes were performed as described in the Materials and methods section using a 0.025 μ M concentration of each protein. Values are means of triplicates, the s.d. being shown by a bar. (a) Hexadecanoyl-CoA; (b) *cis*-9-octadecenoyl-CoA. ■, Bovine hFABP; □, bovine cFABP; ●, bovine ACBP; ○, rat ACBP; △, bovine serum albumin.

independent binding sites for fatty acid and acyl-CoA respectively. However, other investigators have been unable to show the existence of two independent binding sites (Wilkinson & Wilton, 1987; Peeters *et al.*, 1989). In contrast with earlier reports (Ketterer *et al.*, 1976; Mishkin & Turcotte, 1974) hexadecanoyl-CoA was found to bind with lower affinity than hexadecanoic acid to rat and human hFABP (Peeters *et al.*, 1989; Bass, 1985). However, Mishkin & Turcotte (1974) only used gel filtration in the purification of FABP. Their FABP preparation therefore contains ACBP. From the above discussion it might be concluded that FABP has a lower affinity for acyl-CoA than ACBP and that the high affinity of FABP found in previously published experiments most likely is due to ACBP contamination.

It has been shown that hFABP can compete with microsomes (Grinstead *et al.*, 1983) and mitochondria (Barbour & Chan, 1979) for binding of exogenously added acyl-CoA. It has also been shown that acyl-CoA synthetase is stimulated by addition of fatty-acid-binding protein (Wu-Rideout *et al.*, 1976; Burnett *et al.*, 1979; Burrier *et al.*, 1987). Since this enzyme is inhibited by its product, the stimulation by FABP was suggested to be due to release of product inhibition by FABP (Burrier *et al.*, 1987). It has also been shown that FABP protects acyl-CoA against hydrolysis by membrane-bound enzymes (Jamdar, 1979; Burrier *et al.*, 1987).

In none of the above experiments was the purity of the FABP preparations documented. In some of the experiments FABP was only partly purified by gel-filtration chromatography (Jamdar, 1979; Burnett *et al.*, 1979). Some authors have extended purification by negative absorption on a CM-cellulose ion-exchanger (Wu-Rideout *et al.*, 1976; Barbour & Chan, 1979). The former were bound to have had ACBP in their FABP preparations and the latter might have had. Furthermore, it needs a good electrophoretic system with a high gel concentration (20 %, w/v) to separate ACBP and FABP on SDS/polyacrylamide gel, and therefore apparently pure preparations on 10–15 %-polyacrylamide gels might have been contaminated with ACBP. The FABP preparations used in the present study were free of ACBP and vice versa (Fig. 6). We therefore decided to reinvestigate the ability of FABP to extract acyl-CoA esters from

Fig. 6. SDS/polyacrylamide-gel (20 %) electrophoresis of FABP and ACBP

For experimental details, see the Materials and methods section. Lane 1, M_r standards; lane 2, bovine cFABP; lane 3, bovine hFABP; lane 4, bovine ACBP; lane 5, bovine G50 protein; lane 6, rat ACBP, lane 7. The values of M_r standards are indicated by the arrows.

Fig. 7. Effect of bovine hFABP and ACBP on *cis*-9-octadecenoyl-CoA binding to rat liver microsomal membranes

Heated microsomal membranes were incubated at room temperature for 10 min in 85 μ l of 10 mM-Tris/HCl/100 mM-NaCl/5 μ M-*cis*-9-[1- 14 C]octadecenoyl-CoA (sp. radioactivity 7.0 Ci/mol) in the presence or absence (∇) of 5 μ M-bovine hFABP ($\blacksquare$) or -ACBP ($\bullet$). For experimental details, see the Materials and methods section.

biological membranes by FABP preparations shown to be free of ACBP. A further reason to carry out these experiments is that acyl-CoA esters in biological membranes can be either dissolved in the phospholipid bilayer or bound by membrane proteins. The possibility therefore exists that part of the acyl-CoA is bound to low-affinity sites with which both ACBP and FABP can compete.

The results of these experiments (Fig. 7) show that equimolar amounts of FABP can only partly prevent binding of the *cis*-9-octadecenoyl-CoA to heated microsomal membranes. This is in agreement with data reported from experiments with heated microsomes by Grinstead *et al.* (1983). Burrier *et al.* (1987) also showed that rat hFABP could stabilize acyl-CoA synthesized by microsomal acyl-CoA ligases. Again one must have some reservations about the purity of the preparations used in the above experiments. As predicted from the Lipidex and liposome-binding experiment, bovine ACBP was much more effective than bovine hFABP in preventing binding of *cis*-9-octadecenoyl-CoA to microsomal membranes.

In conclusion ACBP binds acyl-CoA esters with much higher affinity than either bovine cFABP or hFABP. As the concentration of ACBP in liver is 2–4-fold the acyl-CoA concentration, it must be expected that ACBP, and not FABP, is the most important transport protein for acyl-CoA in liver. A lot of the previous data on the effect of FABP on acyl-CoA metabolism must be viewed with caution, owing to possible contamination with ACBP.

Note added in proof (received 1 December 1989)

After the present paper was submitted, it was reported that a steroid-synthesis-stimulating protein from bovine adrenal cortex has a sequence identical with that of ACBP (Besman *et al.*, 1989). The only difference between the two proteins is that the adrenal protein is missing the C-terminal Gly-Ile.

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